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THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

SINGLE WALLED CARBON NANOTUBES (SWCNTs): SUMMARY OF THE DOSSIER

**Series on the Safety of Manufactured Nanomaterials
No. 70**

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This document is the summary of the Single Walled Carbon Nanotubes (SWCNTs) dossier, published as:

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OECD Environment, Health and Safety Publications

Series on the Safety of Manufactured Nanomaterials

No. 70

**SINGLE WALLED CARBON NANOTUBES (SWCNTs):
SUMMARY OF THE DOSSIER**

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

**Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris, 2016**

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This summary report includes literature data that take materials into account that differ from the materials tested under the sponsorship programme.

1 IDENTITY

1.1 Identification of the Substance

Chemical name: SWCNTs, Single-Walled Carbon Nanotubes

CAS No.: 308068-56-6

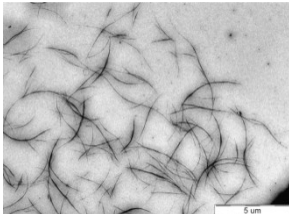
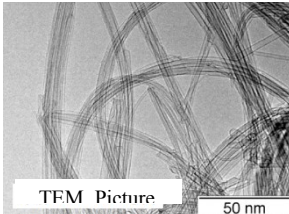
1.2 Purity/Impurities/Additives

Some SWCNTs contain catalytic metals as impurities. Nikkiso SWCNT contains about 4 wt% of Fe and very small amount of other metallic impurities. The purity of Super Growth is >99 wt% (carbon) and it contains very small amount of metals as impurities.

1.3 Physical-Chemical Properties

Table 1 Summary of Physical-Chemical Properties

Endpoint	Principal(1) Nikkiso SWCNT	Principal (2) Super Growth	note
Agglomeration/aggregation	----	particles were in bundle consisting of a few or dozens of SWCNTs. The average bundle length was 0.28 μm (SD=0.17) and the average bundle diameter was 9.3 nm (SD=4.5)	Observed by TEM
Water solubility	insoluble to water	insoluble to water	Niyogi S. et al (2002) ¹
Crystalline phase	G/D = 116 +/- 21	G/D = 9.4 (SD=3.4)	Raman Spectral Analysis The soundness of the structure (Defect of graphene sheet) can be seen by the ratio of G-band and D-band of Raman Spectrum.
Dustiness	n/a	Respirable mass conc. 0.025 mg/m ³	Vortex Shaker Method (Maynard 2004) ²

Single nanotube size	Dia. = 1.86 nm SD = 1.4	Dia. = 3.03 nm SD = 1.11	Observed by TEM
Representative TEM picture			
Particle size distribution	Dia = 2.7 μm SD = 1.4 (measured by DLS)	Dia = 8.2 nm (SD = 1.7) Length = 0.23 μm (SD=1.8) (measured by TEM)	
Specific surface area	877.7 m ² /g	1,064 m ² /g	ISO 9277 (BET method)
Zeta potential	19.9 +/- 5.5 Stable dispersion in Dulbecco's modified Eagle medium with 10% heat-inactivated fetal bovine serum (not described in dossier)	-14.7 +/- 0.9 Stable dispersion in Dulbecco's modified Eagle medium with 10% heat-inactivated fetal bovine serum (not described in dossier)	electrophoretic mobility method
Surface chemistry	O/C = 0.022 (Measured by XPS)	O/C = 0.003 (Measured by XPS)	For checking the absence of functional groups and the defect density
Photocatalytic activity	No photocatalytic activity	No photocatalytic activity	ISO 22197-1 / JIS R 1701
Pour density	n/a (the material is in a felt like form)	0.0192 g/cm ³	ASTM D 1513-05
Porosity	Pore volume =: 30.5 ml/g Davg = 190 nm	Pore volume =: 18.6 ml/g Davg = 54 nm	ISO 15901-1
Octanol-water partition coefficient	n/a	n/a	SWCNTs are insoluble to water
Redox potential			

Radical formation potential including surface properties	---	---	The method is not specified.
--	-----	-----	------------------------------

2 GENERAL INFORMATION ON EXPOSURE

2.1 Environmental Exposure and Fate

2.1.1 Photodegradation

No adequate information is available.

2.1.2 Stability in Water

Bouchard et al.³ studied dispersion stability of SWCNT supplied by **Cheap Tubes Inc.** in water. To determine the long term stability of the SWCNTs in representative environmental systems, the suspended mass concentrations of SWCNTs in 0.01 and 0.001% (w/v) SDS and in 10 mM NaCl, 1.0 mM CaCl₂ and in Calls Creek water were measured over a 6-week period. Column transport studies were conducted under saturated conditions using fresh water sediment. Longer term stability studies demonstrated that SWCNTs remained suspended for over six weeks in surface water. Transport studies in a freshwater sediment showed that SWCNTs retained greater at lower SDS concentrations (0.001%-0.05% w/v) than at a higher SDS concentration (0.1%). These studies demonstrate that low levels of surfactant are effective in stabilizing and mobilizing SWCNTs in environmental media.

2.1.3 Transport between Environmental Compartments

No information is available.

2.1.4 Biodegradation

Six readily bio-degradability tests were conducted with **Nikkiso SWCNT** and **Super Growth** according to OECD test guidelines in compliance with GLP.

Two biodegradation tests according to OECD TG 301F (Manometric respirometry method) were conducted (AIST, 2011^{4,5}). The concentration of **Nikkiso SWCNT** and **Super Growth** were 100 mg/L and the concentration of the activated sludge was 30 mg/L as suspended solid matter. Biodegradation by BOD after 28 day cultivation period was 0 %. Two biodegradation tests according to OECD TG 301C (Modified MITI method (I)) were conducted with cultivation period of 28 days (AIST, 2011^{6,7}). Biodegradation by BOD after 28 day cultivation period was 0 %. Two bio-degradation tests according to OECD TG 302C (Inherent Biodegradability: Modified MITI method (II)) were conducted with cultivation period of 28 days (AIST, 2011^{8,9}). Biodegradation by BOD after 28 day cultivation period was 0 %.

2.1.5 Bioaccumulation

Parks et al.¹⁰ (2012) tested the toxicity, bioavailability, and bioaccumulation of several SWCNTs in marine benthic organisms at the base of the food chain. These SWCNTs included **SG65**, **SG76**, and **CG100** produced by **SouthWest NanoTechnologies** using CoMoCAT method and **14C-radiolabeling SWCNT** produced by **Research Triangle Institute** using the arc discharge method. SWCNTs were

prepared in 2% w/v sodium deoxycholate (SDC, a water soluble bile-acid ionic detergent) using high power sonication at 50% amplitude for 10 minutes in a salt-water ice bath, followed by centrifugation at 17,860 x g and 22°C for 30 minutes to remove non-suspended SWCNT bundles. Toxicity and bioaccumulation tests suspensions of unlabeled SWCNT were prepared at 1 mg SWCNT/ml 2% w/v SDC with no centrifugation. Stock suspensions were resonicated prior to adding to sediment or food sources if not used immediately after preparation.

No significant mortalities were seen in the amphipod *Ampelisca abdita* or the mysid *Americamysis bahia* at measured food- and sediment-borne concentrations of SWCNTs of up to 100 ppm (highest concentration tested) over a seven-day exposure period using static conditions. These experiments were conducted with reference marine sediment (i.e., Long Island Sound) at 0.1, 1, and 10 µg SWCNT/g dry sediment. SWCNTs were not detected in either depurated or non-depurated mysid or amphipod extracts. Additional tests were performed with *Leptocheirus plumulosus* exposed to sediment and/or food (*Isochrysis galbana*, an alga) spiked with ¹⁴C-radiolabeled SWCNTs in 28-day static renewal tests. No significant mortality was observed in this exposure. Sediment and algal concentrations of CNTs were verified at 0 and 28 days. Only in those experiments where high SWCNT amended sediments (100 µg/g sediment + control algae; 100 µg/g sediment + 100 µg/g algae) were presented, elevated body burdens were found (a factor of 5 higher than in controls) following depuration periods of 24 hours. In other treatments (10 µg/g sediment; 10 µg/g dry weight algae) bioaccumulation was limited. Bioaccumulation factors (where BAF = [amphipod]/([sediment]+[algae])) were less than 1, and these decreased following depuration by approximately one order of magnitude.

The data indicate that these SWCNTs are not bioaccumulative: benthic organisms take up the SWCNTs through ingestion and then rapidly eliminate them during depuration (radioactivity measured in fecal pellets, where SWCNTs added to sediment or food was significant relative to controls). Additional research is needed to examine what occurs when organisms with a gut burden of CNTs is ingested by a predator: the guts of some marine species contain surfactants similar to SDC and gum arabic making the SWCNTs more bioavailable for gut uptake into a predator's tissues. In this study, measured concentrations of several SWCNTs in water, food, tissues, and sediments were conducted using a novel near infrared fluorescence (NIRF) spectroscopic method. The NIRF methodology is based on each SWCNT having a unique diameter, chiral wrapping angle, and excitation-emission spectrum. The resulting unique spectral signature allows for identification of individual types of SWCNTs. This methodology, in its automated form and using deoxycholic acid sodium salt (SDC) for the extraction and resuspension of SWCNTs from sediments, water, and tissues, is available in Schierz et al. ¹¹(2012).

Diamond et al. ¹² studied bioaccumulation of SWCNT supplied by SWeNT in *Amperisca abdita* and *Americamysis bahia*. *Cyclotella sp* (algae) and *Artemia salina* (brine shrimp) were spiked at 1 µg/g of SWCNT, respectively, as prey for *Amperisca abdita* and *Americamysis bahia*. Trophic transfer was measured using novel near infrared fluorescence (NIRF) spectroscopic method. No bioaccumulation of SWCNT was observed.

Summary

Biodegradation

Nikkiso and Super Growth showed no biodegradation through various tests like OECD TG301C, TG301F and TG302C using epipelagic water of rivers, lakes and inland seas or sludge from waste water treatment plant.

Bioaccumulation

The data on SWCNTs SG65, SG76, and CG100 produced by SouthWest NanoTechnologies indicate that these SWCNTs are not bioaccumulative.

3 HAZARDS TO THE ENVIRONMENT

The studies summarized below provide an overview of the adverse effects of SWCNTs that have been reported in the literature. Direct application of most of these study results to the OECD principle or alternate SWCNTs has to be considered in the context of the similarities and differences between the physical chemical properties of the materials used in the studies below. This review focuses on underivatized SWCNTs, since these are most directly relevant to the OECD SWCNTs. However, it should be noted that, depending on the modes of action of CNT toxicity, effects seen with other forms of CNTs and CNFs may be relevant. Assessment in this report is focused on the products which may contain impurities.

3.1 Aquatic Effects

Acute Toxicity Test Results

Fish

Available data on the acute toxicity to fish are summarized in Table 2-1.

An acute toxicity test of **Nikkiso SWCNT** was conducted for Japanese Medaka, *Oryzias latipes* according to OECD TG 203 in compliance with GLP (AIST, 2011¹³). Ten fish per concentration were exposed in static system renewed daily at nominal concentrations of 10 mg/L with a blank control, in 24 +1°C. The measured mean concentrations were determined as time weighted mean from analytical measured values using a GC/MS at time ranging from 0-96 hours. No mortality was observed at the concentrations of 10 mg/L. The 96h LC₅₀ was determined at >10 mg/L.

An acute toxicity test of **Super Growth** was conducted for Japanese Medaka, *Oryzias latipes* according to OECD TG 203 in compliance with GLP (AIST, 2011¹⁴). Ten fish per concentration were exposed in static system renewed daily at nominal concentrations of 10 mg/L with a blank control, in 24 +1°C. The measured mean concentrations were determined as time weighted mean from analytical measured values using a GC/MS at time ranging from 0-96 hours. No mortality was observed at the concentrations of 10 mg/L. The 96h LC₅₀ was determined at >10 mg/L.

Cheng et al.¹⁵ (2007) studied the effects of **SWCNTs** on developing zebrafish (*Danio rerio*) embryos according to OECD TG 236. SWCNTs (produced by arc discharge in the presence of Co and Ni as catalysts, then purified by acid leaching and thermal oxidation) were obtained from **Sigma-Aldrich** and had the following characteristics according to the supplier: 11 nm average diameter, 0.5 – 100 um average length, purity of 90 atom percent. The SWCNTs were covered with negatively charged carboxylic acid grouped at the defect sites on their sidewalls, and still had cobalt and nickel present that were not encapsulated. SWCNTs were dispersed in filtered tap water by stirring with a magnetic stirrer bar for 30 minutes, generating six concentrations of SWCNTs (20, 40, 120, 240, and 360 mg/L). Carbon black and dilution water controls were also tested, and embryos were exposed from 4 to 96 hpf (hours post fertilization). The embryos were examined at 24, 48, 52, 56, 72, 75 and 96 hpf. SEM images revealed that most of the SWCNT agglomerates tested were micron-sized, while the zebrafish chorion pore size was much smaller (nanometer sized): the chorion may have served as an effective barrier to CNT penetration. Delayed hatching was found at concentrations of 120 mg/L and higher, but 99% of the exposed embryos hatched by 75 hpf. Delayed hatching cannot be denied by cobalt and nickel catalyst contaminants used in manufacture: nominal no-effect concentrations for hatching time were 40 µg/L for Ni and 3,840 µg/L for Co in referenced studies; concentrations were estimated (based on manufacturer's impurity descriptors) to be greater than 3 mg/L Ni and 9 mg/L Co in the study by Cheng, et al. (2007).

Canada¹⁶ conducted three acute toxicity studies of SWCNT supplied by **NRC** according to OECD Fish Embryo Toxicity Test (TG 236) using zebrafish. The first study was done at the concentration of 200 mg/L for 96 hours exposure period, the second study was done at the concentration of 1, 10 and 100 mg/L

for 96 hours exposure period, the third experiment was done at the concentration of 1, 10, 50, 100 and 200 mg/L for 72 hours exposure period. Either experiment showed no significant change in mortality, morphology and behaviour.

Smith et al.¹⁷ exposed juvenile rainbow trout to SWCNTs at concentrations of 0, 0.1, 0.25, or 0.5 mg/L. SWCNTs were brought into the aqueous phase through the use of the detergent-like solvent sodium dodecyl sulfate (SDS) and sonication. SWCNTs were obtained from Cheap Tubes, and had the following characteristics according to the supplier: 1.1 nm mean outside diameter, 5-30 μ m length, minimum 96.3% carbon; maximum impurities were Al 0.08, CL 0.41, Co 2.91, and S 0.29%. Fish were exposed for up to 10 days under semi-static conditions. Stock solution concentrations of SWCNTs were confirmed, and re-dosing with SWCNT-bearing water occurred every 12 hours (in-tank concentrations of SWCNTs could not be determined). A variety of endpoints were monitored including clinical observations, clinical chemistry, differential blood analysis, and histopathology. Effects on the gill and brain were found that may be related to the accumulation of SWCNTs on gills (inhibiting oxygen uptake) and/or to the sodium dodecylsulfate vehicle interaction with SWCNTs and fish tissue. A dose-dependent relationship of increasing ventilation rate was seen with increasing SWCNT concentrations from 0.1 to 0.25 to 0.5 mg/L, relative to an SDS control. In a related observation, effects on gill morphology were seen at all three concentrations (relative to both the solvent and water-only control individuals), but there was not a clear dose-response relationship between gill morphology alterations and dose. A third line of evidence points to respiratory toxicity: fish showed signs of gill irritation and mucus secretion at all SWCNT-SDS doses, but not in the solvent or water-only controls. By day 4 there were strands of sloughed mucus, and elevated mucus clearly visible on the gills. While this study was very well conducted, it is difficult to attribute the effects seen solely to the SWCNTs (ruling out effects due to SWCNT-SDS combined effects): concentrations of SDS were well below those which would cause toxicity to trout, and SDS-only controls showed no adverse effects on respiratory toxicity endpoints. However, the effects of SDS on SWCNT physical-chemical properties (and any resultant effects on adverse effects) may not be fully understood at this time.

Fourteen days prolonged toxicity test of **Nikkiso SWCNT** was carried out with Japanese Medaka, *Oryzias latipes* according to OECD TG 204 under flow-through conditions (AIST, 2011¹⁸). Test parameters included fish growth (weight and length). The fish weights at the start of the experiment varied between 0.057 and 0.099 g. Nominal concentrations ranged from 0.10 to 10 mg/L. Concentrations were measured at the start, halfway through and at the end of the test period. Survival of the fish was not significantly affected up to and including 10 mg/L. The 14-d EC₅₀, 14-d NOEC and 14-d LOEC was >10 mg/L, ca. 10 mg/L and >10 mg/L, respectively.

Fourteen days prolonged toxicity test of **Super Growth** was carried out with Japanese Medaka, *Oryzias latipes* according to OECD TG 204 under flow-through conditions (AIST, 2011¹⁹). Test parameters included fish growth (weight and length). The fish weights at the start of the experiment varied between 0.057 and 0.099 g. Nominal concentrations ranged from 0.10 to 10 mg/L. Concentrations were measured at the start, halfway through and at the end of the test period. No significant effects on mortality, growth inhibition and toxicological symptoms were observed up to 10 mg/L. The 14-d EC₅₀, 14-d NOEC and 14-d LOEC was >10 mg/L, ca. 10 mg/L and >10 mg/L, respectively.

Table 2-1. Acute Toxicity to Aquatic Vertebrates

Test Material	Species	Duration	NOEC (mg/L, unless otherwise noted)	LOEC (mg/L)	LC50 (mg/L)	Authors , Year, others
Nikkiso	<i>Oryzias latipes</i>	96 hours			>10	AIST, 2011 TG203

Super Growth	<i>Oryzias latipes</i>	96 hours			>10	AIST , 2011 TG203
SWCNTs from Sigma-Aldrich	Zebrafish (<i>Danio rerio</i>) embryo	4 to 96 hours post fertilization		120		Cheng et al., 2007 TG236
Nikkiso	<i>Oryzias latipes</i>	14 days	10	>10	>10	AIST, 2011 TG204
Super Growth	<i>Oryzias latipes</i>	14 days	10	>10	>10	AIST , 2011 TG204
Cheap Tubes	Rainbow Trout (<i>Salmo gairdnerii</i>)	10 days	respiratory toxicity Gill irritation and mucus secretion at 0.1 to 0.5 mg/L			Smith et al. , 2007

Invertebrates

Available data on the acute toxicity to aquatic invertebrates are summarized in Table 2-2.

Zhu et al.²⁰ (2009) examined the acute toxicity of SWCNTs using the same as manufactured SWCNTs as Mwangi et al (2012) and followed the OECD TG 202 (with modifications in accordance with the OECD guidance document for difficult to test substances²¹). The **SWCNTs** were produced by **Shenzhen Nanotech Port Co., Ltd.** and had the following information supplied from the manufacturer: diameter < 2 nm, length 5-15 um, purity: SWCNT > 60, CNT > 90%. A stock solution was prepared in reconstituted water using vigorous shaking at room temperature (specifics not provided) to a nominal concentration of 1,000 mg/L; dilutions of the stock were used for the 48-hour exposures of daphnids (0.1, 0.5, 1, 5, 10, 50, and 100 mg/L). Beakers containing test organisms were shaken constantly at 140 rpm, and daphnids were not fed during the test. A dose-dependent increase in toxicity was seen with increasing nominal SWCNT concentrations, with both immobilization and mortality assessed. Large amounts of SWCNTs were found in the gut of the daphnids.

Daphnia magna were exposed to **Nikkiso SWCNT** at nominal concentrations of 0, 0.42, 0.94, 2.1, 4.5 and 10 mg/L for 48 hours in a static system according to OECD TG 202. Daphnids were obtained from University of Sheffield and maintained over 10 years at the laboratory, and twenty daphnids per concentration within 24 hr old were used for the test. Immobility was 0, 0, 0, 0, 5, 0 and 25 % after 48 hours. The 48-hour EC₅₀ was >10 mg/L based on the arithmetic mean of measured concentrations (AIST, 2011²²).

Daphnia magna were exposed to **Super Growth** at nominal concentrations of 0, 0.42, 0.94, 2.1, 4.5 and 10 mg/L for 48 hours in a static system according to OECD TG 202. Daphnids were obtained from University of Sheffield and maintained over 10 years at the laboratory, and twenty daphnids per concentration within 24 hr old were used for the test. Immobility was 0, 0, 0, 0, 5, 0 and 25 % after 48 hours. The 48-hour EC₅₀ was >10 mg/L based on the arithmetic mean of measured concentrations (AIST, 2011²³).

Table 2-2. Acute Toxicity to Aquatic Invertebrates

Test Material	Species	Duration	Other	Authors, Year, others
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Nikkiso	<i>Daphnia magna</i>	48 hours	EC ₅₀ >10 mg/L	AIST, 2011 OECD TG 202
Super Growth	<i>Daphnia magna</i>	48 hours	EC ₅₀ >10 mg/L	AIST, 2011 OECD TG 202
Shenzhen Nanotech Port Co., Ltd.	<i>Daphnia magna</i>	48 hours	EC ₅₀ = 1.306 mg/L (immobilization)	Zhu et al. (2009) Modified TG 202

Aquatic plants (e.g algae)

Available data on the acute toxicity to aquatic plants are summarized in Table 2-3.

Youn et al.²⁴ conducted 96-hour growth inhibition tests with *Pseudokirchneriella subcapitata* using nominal concentrations of SWCNTs and an adaptation of a standardized US EPA protocol (Method 1003.0). Raw SWCNTs (**Rice HPR 145.1**) were examined: they had a length distribution between 300-1200 nm, and contained metal impurities of 13.4%, or 17 ppm following preparation of stock solutions in gum arabic. The study employed gum arabic at two different concentrations. SWCNTs were dispersed into stock solutions using gum arabic at 1%, followed by homogenization with a high shear mixer for 1.5 hours and ultrasonication for 10 minutes. The mixture was then ultra-centrifuged at 20,000 rpm for 2.5 hrs and the supernatant separated from the aggregated SWCNTs at the bottom of the centrifuge tube. The LOEC and NOEC concentrations were obtained in test media with final gum arabic concentrations of 0.023% v/v. In contrast, a second study conducted in an identical fashion with a higher concentration of gum arabic (0.0023 – 0.0046% v/v) found no growth inhibition at test concentrations up to 0.5 ppm. Literature was cited that indicated that gum arabic is nontoxic to algae, unlike other surfactants such as sodium dodecyl sulfate.

Using “small scale” bioassays with a variety of organisms and cells (algae, microorganisms, fish hepatocytes, & invertebrates), Blaise et al.²⁵ (2008) found growth inhibition in *Pseudokirchneriella subcapitata* at a reported nominal concentration of 1.04 mg/L, and found no effects for other organisms. SWCNTs were obtained from **Sigma-Aldrich (no. 519308)**: they were characterized by the supplier as 50-70% SWCNTs, in bundles with diameter X length dimensions as (1.2. – 1.5 nm) X (2-5 um). A stock solution of 500 ml/L of SWCNTs (nominal concentration) was prepared in purified water by rotator mixing at room temperature (24 hours at 12 rpm), and then filtered through a 0.22 um cellulose membrane. Actual concentrations tested on algae were not reported.

For a freshwater algal species, *Pseudokirchneriella subcapitata*, the toxicity of **Nikkiso** SWCNT was studied (AIST, 2011²⁶). An algal growth inhibition test (OECD TG 201) was carried out in compliance with GLP. Nominal concentration in the test water was 0, 0.10, 0.32, 1.0, 3.2 and 10 mg/L, respectively. Concentration related growth inhibitions were observed and the 72h EC₅₀ and 72h NOEC were determined as >10 and ca. 0.32 mg/L, respectively.

For a freshwater algal species, *Pseudokirchneriella subcapitata*, the toxicity of **Super Growth** was studied (AIST, 2011²⁷). An algal growth inhibition test (OECD TG 201) was carried out in compliance with GLP. Nominal concentration in the test water was 0, 0.10, 0.32, 1.0, 3.2 and 10 mg/L, respectively. Concentration related growth inhibitions were observed and the 72hEC₅₀ and 72h NOEC were determined as >10 and ca. 0.32 mg/L, respectively.

Table 2-3. Acute Toxicity to Aquatic Plants

Test Material	Species	Duration	NOEC (mg/L)	LOEC (mg/L)	LC ₅₀ (mg/L)	Other	Authors, Year, others
Nikkiso	Green alga (<i>Pseudokirchneriella subcapitata</i>)	72 hours	0.32			EC50>10 mg/L	AIST 2011 OECD TG 201
Super Growth	Green alga (<i>Pseudokirchneriella subcapitata</i>)	72 hours	0.32			EC50>10 mg/L	AIST, 2011 OECD TG 201
Rice HPR 145.1	Green alga (<i>Pseudokirchneriella subcapitata</i>)	96 hours	0.05	0.25			Youn, S et al., 2011 US EPA protocol (Method 1003.0)
Sigma-Aldrich (no. 519308)	Green alga (<i>Pseudokirchneriella subcapitata</i>)	72 hours				IC ₂₅ = 1.04 mg/L for growth inhibition	Blaise et al., 2008

Chronic Toxicity Test Results

Fish

Data on the chronic toxicity to aquatic vertebrates are summarized in Table 2-4.

Fraser, et al.²⁸ exposed juvenile female trout to SWCNTs in feed at a concentration of 500 mg/kg for six weeks and monitored for mortality, growth, blood parameters, tissue alterations, and changes in biochemical parameters. The SWCNTs were obtained from **Cheap Tubes**, and had the same characteristics as those used in Smith, et al. (2007). Dietary exposure to SWCNTs had no significant effect on growth, hematology, or other measured endpoints in trout. Lipid peroxidation, as indicated by changes in thiobarbituric acid reactive substance (TBARS) measurements, was elevated in the brain which suggested that lipid peroxidation was a consequence of exposure to CNTs. However, the levels of TBARS which were elevated at week 4 did not differ significantly from brain TBARS measured in control fish at other time points. It was noted that longer duration studies, other fish species, and additional endpoints (such as bioenergetics and swimming speed) have not been examined.

Table 2-4 Chronic Toxicity to Aquatic Vertebrates

Test Material	Species	Duration	NOEC (mg/L, unless otherwise noted)	LOEC (mg/L)	LC50 (mg/L)	Authors, Year, others
Cheap Tubes	Rainbow Trout (<i>Salmo gairdnerii</i>), multiple endpoints	6 weeks	No effect of feeding 500 mg-CNT/kg-food except for elevation of TBARS measurement in brain			Fraser et al., 2011

Aquatic Invertebrates

Available data on the chronic toxicity to aquatic invertebrates are summarized in Table 2-5.

Mwangi et al²⁹ examined the effects of SWCNTs (the SWCNTs used were the same as described in Zhu, et al., 2009) on sediment-dwelling invertebrates in 14-day tests adapted from ASTM standard test protocols E1706-05 and E729-96, and each 200 ml volume of test solution containing the organisms and CNTs also contained 5 ml of fine sand. A single concentration of 1,000 mg/L SWCNTs was used, and only nominal concentrations were reported for the static tests (run with feeding, and replenishment of non-laden CNT water only). Two SWCNT stock solutions were prepared: one with sonication (2 min at 65 W) and stirring, and the other with only stirring; no other dispersion agents were used. Significant differences in body weight and survival were noted for amphipods, midges and oligochaetes relative to the plain water control; sonicated SWCNTs had significantly higher adverse effects than unsonicated SWCNTs.

A chronic toxicity test of **Nikkiso SWCNT** with *Daphnia magna* was conducted at 0.0030, 0.0095, 0.030, 0.095 and 0.30 mg/L under semi-static conditions (water renewal: every 2 days) for 21 days according to OECD TG 211. NOEC values in this chronic toxicity test to *Daphnia magna* on reproduction were ca. 0.3 mg/L (TASC, 2011³⁰).

A chronic toxicity test of **Super Growth** with *Daphnia magna* was conducted at 0.01, 0.032, 0.10, 0.32 and 1.0 mg/L under semi-static conditions (water renewal: every 2 days) for 21 days according to OECD TG 211. NOEC values in this chronic toxicity test to *Daphnia magna* on reproduction were ca. 0.32 mg/L (TASC, 2011³¹).

Templeton et al³² (2006) found no significant effects in full life-cycle studies on survival/developmental/reproductive endpoints with the estuarine meiobenthic copepod *Amphiascus tenuiremis* exposed to chemically purified arc-discharge produced SWCNTs at sedimentary concentrations of 0, 0.58, 0.97, or 1.5, or 10 mg/L (presumed to be nominal concentrations). SWCNTs were produced by a bench-scale arc discharge method, and purified by reflux in 3.3 N nitric acid (which removed metallic and carbonaceous contaminants). This chemical process oxidized the SWCNTs at their termini and defect locations, and incorporated hydroxyl and carboxylic acid functional groups (which increased SWCNT water solubility). Tests were conducted according to a standardized ASTM protocol (ASTM E-2317-04), in 200 ul microplate assays where media were replaced every 4 days. “As prepared” or unpurified SWCNTs with metallic contaminants showed significant toxicity at 10 mg/L relative to survival, developmental success, development rate, and fertilization success. Interestingly, an extract of the unpurified SWCNTs (which contained small fluorescent nanocarbon byproducts and no metallic impurities) resulted in significantly increased life cycle mortality at 10 mg/L, and caused significant reduction in life cycle molding success even at the lowest concentration tested (0.58 µg/L). This fluorescent fraction was fully soluble in aqueous test solutions; its particles were less than 18 nm in length, with equal widths and heights near 1 nm.

Table 2-5 Chronic Toxicity to Aquatic Invertebrates

Test Material	Species	Duration	NOEC (mg/L)	LOEC (mg/L)	LC50 (mg/L)	Other	Authors, Year, others
Nikkiso	<i>Daphnia magna</i>	21 days	0.3	>0.3	>0.3	EC50>0.3 mg/L	TASC, 2011 OECD TG 211
Super Growth	<i>Daphnia magna</i>	21 days	ca. 0.32	ca. 1	ca. 0.62	EC50>1 mg/L	TASC, 2011 OECD TG 211
Shenzhen Nanotech Port Co.,	Sediment dwelling amphipod	14 days	80% mortality at 1,000 mg/L (unsonicated CNTs); 100% mortality (sonicated CNTs)				Mwangi et al., 2012

Ltd.	(<i>Hyalella azteca</i>)		0% mortality (controls)				
Shenzhen Nanotech Port Co., Ltd.	Sediment dwelling midge (<i>Chironomus dilutus</i>)	14 days	90% mortality at 1,000 mg/L (unsonicated CNTs); 100% mortality (sonicated CNTs); 17% mortality (controls)				Mwangi et al., 2012
Shenzhen Nanotech Port Co., Ltd.	Sediment dwell oligochaete (<i>Lumbriculus variegatus</i>)	14 days	Biomass (mg) 1.4 (unsonicated CNTs); 2.8 (sonicated CNTs); 3.7 (controls)				Mwangi et al., 2012
Chemically purified SWCNT by bench-scale arc-discharge method	Marine and estuarine sediment dwelling copepod (<i>Amphiascus tenuiremis</i>)	Life Cycle durations of 28 to 35 days	1.6 mg/L for as prepared SWCN Ts	10 mg/L for as prepared SWCN Ts			Templeton et al., 2006
Chemically purified SWCNT by bench-scale arc-discharge method	Marine and estuarine sediment dwelling copepod (<i>Amphiascus tenuiremis</i>)	Life Cycle durations of 28 to 35 days	10 mg/L for purified SWCN Ts				Templeton et al., 2006

Effects on activated sludge at WWTP

Available data on the effects on activated sludge at WWTP are summarized in Table 2-6.

The toxicity of **Nikkiso SWCNT** to activated sludge was evaluated by a simple respirometric procedure set up on the basis of OECD TG 209. A 3h EC₅₀ value of >100 mg/L was derived from this study (AIST, 2011³³).

The toxicity of **Super Growth** to activated sludge was evaluated by a simple respirometric procedure set up on the basis of OECD TG 209. A 3h EC₅₀ value of >100 mg/L was derived from this study (AIST, 2011³⁴).

Table 2-6 Effects on activated sludge

Test Material	Species	Duration	NOEC (mg/L)	LOEC (mg/L)	LC50 (mg/L)	Other	Authors, Year, others
Nikkiso	Activated sludge	3 hours				EC ₅₀ >100mg/L	AIST, 2011 OECD TG 209
Super Growth	Activated sludge	3 hours				EC ₅₀ >100mg/L	AIST, 2011 OECD TG 209

3.2 Terrestrial Effects

Available data on the terrestrial effects are summarized in Table 2-7.

Canas, et al.³⁵ (2008) studied the effects of SWCNTs on root elongation in six crop species recommended by the US EPA for phytotoxicity studies. **P3-SWCNTs** were purchased from **Carbon Solutions, Inc**

and had the following characteristics according to the manufacturer: 80-90% purity, 3% metal content by weight. CNT suspensions were prepared by mixing 100 mg of CNTs with 4 ml of water and then sonicating for 30 min. Then, an additional 16 ml of water was added, and sonication continued for an additional 90 min. to provide the stock solution. Dilutions of this stock solution with water were made to produce test concentrations of 56, 315, and 1,750 mg/L of CNTs (nominal concentrations). A water-only control was also run, and each plant (which was grown from seed in the absence of CNTs until most generated radicals) was exposed to 1 ml of test solution in a petri dish with filter paper, and examined at 24 and 48 hours. Regressions were run comparing root elongation to CNT concentrations over the two time courses. Significant decreases in root elongation were seen in tomato plants at both 24 and 48 hrs; significant increases in root elongation were seen in onions at 24 and 48 hrs, and a significant increase in root elongation was seen in cucumbers at 24 hrs. Electron microscopy showed extensive CNT sheets covering root surfaces which could affect necessary plant and root functions that would not be evident in root elongation tests of this duration.

Liu, et al.³⁶ (2009) examined the ability of SWCNTs to penetrate *Nicotinana tabacum L.cv. bright Yellow 2* (BY-2) cells in vitro. Water-soluble SWCNTs were prepared from **Hipco SWCNTs (Carbon Nanotechnologies, Inc.**; length < 500 nm). SWCNTs were oxidized (concentrated sulfuric acid/nitric acid mixture; ultrasonication for 24h, filtration, and further sonication), and labeled noncovalently with fluorescein isothiocyanate (by centrifugation and sonication). Cells were treated with an estimated 2, 10, 20, 40, and 80 mg/L of labeled SWCNTs for 2 hours at 26°C or 4°C. Cells showed increasing penetration of cell walls and membranes with increasing concentrations of SWCNTs at 26°C. Cells showed little uptake of SWCNTs at 4°C, indicating the involvement of endocytosis as a mechanism for penetration.

The toxicity of **Nikkiso SWCNT** to soil microorganisms was evaluated on the basis of OECD TG 216. A 28d EC50 value of >1000 mg/kg soil dw was derived from this study (AIST, 2011³⁷).

The toxicity of **Super Growth** to soil microorganisms was evaluated on the basis of OECD TG 216. A 28d EC50 value of >1000 mg/ kg soil dw was derived from this study (AIST, 2011³⁸).

Table 2-7 Terrestrial Effects

Test Materials	Species	Duration	NOEC (mg/L)	LOEC (mg/L)	LC50 (mg/L)	Other	Authors, Year, others
Nikkiso	Soil (Microorganisms)	28 days				EC50>1000 mg/kg soil dw	AIST, 2011 OECD TG 216
Super Growth	Soil (Microorganisms)	28 days				EC50>1000 mg/kg soil dw	AIST, 2011 OECD TG 216
P3-SWCNTs from Carbon Solutions, Inc	six crop species	24, 48 hours				Decrease in root elongation; tomato (24,48 h)Increase ; onion (24,48h)and cucumber(24h)	Canas et al., 2008
HiPco SWCNTs (Carbon Nanotechnologies, Inc.)	<i>Nicotinana tabacum L.cv. bright Yellow</i> (BY-2) cells	2 hours				Cells showed increasing penetration of cell walls and membranes with increasing concentrations of SWCNTs	Liu et al., 2009

3.3 Summary

Acute toxicity

Acute aquatic toxicity data are available for several SWCNTs. For fish, 96-hour LC₅₀ values are > 10 mg/L for **Nikkiso** and **Super Growth** by the tests according to OECD TG 203 using Japanese Medaka. The test using Zebrafish embryo according to OECD TG 236 suggests 4-96 hours post fertilization LOEC is 120 mg/L for **SWCNTs from Sigma Aldrich** but the effect of contaminant cannot be denied. For daphnids, 48-hour EC₅₀ values are > 10 mg/L for **Nikkiso** and **Super Growth**, and 1.306 mg/L for **SWCNTs from Shenzhen Nanotech** by the tests according to OECD TG 202. For algae, 72-hour NOECs are > 10 mg/L for both **Nikkiso** and **Super Growth** by the tests according to OECD TG 201. 96 hours NOEC for **Rice HPR 145.1** is 0.05 mg/L by the test following US EPA protocol (Method 1003.0).

Prolonged aquatic toxicity data are available for several SWCNTs. For fish, values of 14-day NOEC are 10 mg/L for both **Nikkiso** and **Super Growth** by the tests according to OECD TG 204 using *Oryzias latipes*. In 10 days respiratory toxicity test, Rainbow trout showed Gill irritation and mucus secretion at 0.1 to 0.5 mg/L of **Cheap Tubes SWCNTs**.

Chronic toxicity

In a 6 weeks dietary exposure test using *Salmo gairdnerii*, feeding of **Cheap Tubes SWCNTs** at a concentration of 500 mg/kg-food showed no effect except for elevation of TBARS measurement in brain.

For daphnids, 21-day NOECs are 0.3 mg/L for **Nikkiso** and 0.32 mg/L for **Super Growth** by the test according to OECD TG 211. In 14 days test using invertebrates, mortality at 1,000 mg/L of **Shenzhen Nanotech Port SWCNT** are 80% without sonication and 100% with sonication for *Hyalella Azteca*, and 90% without sonication and 100% with sonication for *Chironomus dilutes*. In the life time test using *Amphiascus tenuiremis* with **SWCNT by bench-scale arc-discharge method**, NOECs are 1.6 mg/L for as prepared SWCNTs and 10 mg/L for purified SWCNTs.

Effects on activated sludge

Based on OECD TG 209, values of 3-hour EC₅₀ are > 100 mg/L for both **Nikkiso** and **Super Growth**.

Terrestrial Effects

Based on OECD TG 216, values of 28 days EC₅₀ are > 1000 mg/kg soil dw for both **Nikkiso** and **Super Growth**. Effects of **P3-SWCNTs from Carbon Solution Inc.** on root elongation of six crop species were tested. Decrease in root elongation was seen for tomato (24 and 48 hours) and increases in root elongation were seen for onion (24 and 48 hours) and cucumber (24 hours). The penetration of **Hipco SWCNTs from Carbon Nanotechnologies, Inc.** was tested using *Nicotinana tabacum L.cv. bright Yellow-2*. Cells showed increasing penetration of cell walls and membranes with increasing concentrations of SWCNTs at 26C, but little uptake at 4C.

4 HUMAN HEALTH HAZARDS

4.1 Effects on Human Health

4.1.1 Toxicokinetics

Studies in Animals

In vivo Studies

The distribution of hydroxylated SWCNT with radioactive ^{125}I atoms (^{125}I -SWNTols) was investigated in male KM mice (Wang et al.³⁹ 2004). Mice were administered ^{125}I -SWNTols by four modes: intraperitoneal (i.p.), subcutaneous injection, gavage, or intravenous (i.v.). After i.p. administration, ^{125}I -SWNTols moved easily among the compartments and tissues of the body except in brain. ^{125}I -SWNTols accumulate readily and are retained for long time periods in the bone. About 80% of ^{125}I -SWNTols are excreted from urine (94%) and feces (6%) within 11 days. The distribution was not markedly influenced by the administration modes.

Cherukuri et al.⁴⁰ (2006) monitored SWCNT intravenously administered to New Zealand White rabbits at 75 μg (ca. 20 $\mu\text{g}/\text{kg}$ bw). The characteristic near-infrared fluorescence spectra indicated that blood proteins displaced the nanotube coating of synthetic surfactant molecules within seconds. The SWCNT concentration in the blood serum decreased exponentially with a half-life of 1.0 +/- 0.1 h. No adverse effects from low-level SWCNT exposure could be detected from behavior or pathological examination. At 24 hours after intravenous administration, SWCNTs were found only in the liver using the optical detection method.

Studies in Humans

No information is available.

Summary

After i.p. administration of ^{125}I -SWNTols in male mice, ^{125}I -SWNTols moved easily among the compartments and tissues of the body except in brain. ^{125}I -SWNTols accumulate readily and are retained for long time periods in the bone. About 80% of ^{125}I -SWNTols are excreted from urine (94%) and feces (6%) within 11 days. The distribution was not markedly influenced by the administration modes (intraperitoneal, subcutaneous injection, gavage, or intravenous). After i.v. administration of SWCNT in rabbits, the SWCNT concentration in the blood serum decreased exponentially with a half-life of 1.0 +/- 0.1 h. At 24 hours after administration, significant concentrations of SWCNTs were found only in the liver.

4.1.2 Acute Toxicity

Studies in Animals

Inhalation

An inhalation exposure to SWCNT (CNI, HiPco) in C57BL/6 mice (4 days, 5h/day at $5.52 \pm 1.37 \text{ mg}/\text{m}^3$) resulted in qualitatively similar pulmonary reaction as pharyngeal aspiration (0-20 $\mu\text{g}/\text{mouse}$). However, SWCNT inhalation was more effective than aspiration in causing inflammatory response, oxidative stress, collagen deposition, and fibrosis as well as mutations of *K-ras* gene locus in the lung of mice (Shvedova et al.⁴¹ 2008; NIOSH).

Intratracheal

A single dose of **Nikkiso SWCNT** by intratracheal instillation was conducted in male Wistar rats (20 rats/group) at 0.2 mg/rat (c.a. 0.67 mg/kg bw) or 0.4 mg/rat (c.a. 1.33 mg/kg bw) (NEDO [#NN-14] unpublished study). Inflammation and fibrosis were examined from the third day to six months after instillation. Both dose groups showed inflammatory cellular infiltration, and particularly it lasted for six months for high dose. Also, heme oxygenase-1 (HO-1) gene expression increased continuously in both dose groups. Observation is ongoing up to two years.

Kobayashi et al.⁴² (2012) reported two intratracheal studies conducted by NEDO project [#NN-05-1 and #NN-05-2]. Male SD rats were given **Super Growth SWCNT** by intratracheal instillation at 0.2 or 2 mg/kg, and observation was carried out from 24 h to 3 months after administration (1st study). Male SD rats were given **Super Growth SWCNT** by intratracheal instillation at 0.04, 0.2 or 1 mg/kg and observation was carried out from 3 days to 6 months after administration. There were dose-dependent inflammatory responses in the lungs in both studies. Significant increases in pulmonary tissue inflammation and inflammatory biomarkers persisted at 1 and 2 mg/kg for 6 months.

Male SD rats were exposed to SWCNT (CNI, HiPco) by intratracheal instillation at 0.2 or 1 mg/kg and observed for 3 months (NEDO [#NN-10] unpublished study). There was a dose-dependent inflammatory response in the lungs. The inflammation was recovered by one month after instillation at 0.2 mg/kg, but not at 1 mg/kg.

Male Crl:CD(SD) rats were exposed to **Nikkiso SWCNT** (NEDO [#NN-17]), **Super Growth SWCNT** (NEDO [#NN-15] unpublished study) and Meijo SWCNT (NEDO [#NN-17]) (NEDO [#NN-16] unpublished study) by intratracheal instillation. SWCNT suspensions were prepared with 3 types of methods. Four weeks after instillation, inflammation effects in the lung are Super Growth SWCNT > Nikkiso SWCNT > Alternate Meijo SWCNT (suspended in 0.2% DCA with 1% Tween 80). Both high and low doses showed inflammatory cellular infiltration and it lasted for six months for high dose.

The following data are not for principal SWCNT.

Warheit et al.⁴³ (2004) investigated the acute lung toxicity of intratracheally instilled SWCNTs (DuPont; 1.4 nm diameter; >1µm length) in male Crl:CD(SD)IGS BR rats. Exposures to SWCNTs caused death (about 15% of rats) within 24 h postinstillation at 5 mg/kg bw, but it was considered to be due to mechanical blockage of the upper airways and was not due to inherent pulmonary toxicity of SWCNT. No death was observed at 1 mg/kg bw, and no abnormality was observed in eating behavior and weight gain in animals that survived at 1 and 5 mg/kg bw throughout the duration of the study (3 month). Exposures to SWCNT produced transient inflammatory and cell injury effects.

Lam et al.⁴⁴ (2004) investigated the acute lung toxicity through intratracheal instillation test to male B6C3F mice using three different SWCNTs [1: **Rice Univ, HiPco**; 2: **Rice Univ, refined HiPco**; 3: **CaboLex**] made by different processes and containing different types and amount of residual catalytic metal at doses of 0, 0.1 and 0.5 mg/animal. All nanotube products induced dose-dependent epithelioid granulomas. In some cases, interstitial inflammation, peribronchial inflammation and necrosis that had extended into the alveolar septa were observed.

ICR mice were exposed to SWCNT (unknown identification) by intratracheal instillation at 0.5 mg/kg (Chou et al.⁴⁵, 2008). Foamy macrophages phagocytosing CNT were observed at 3 days post-instillation and multifocal granuloma and foamy macrophages at 14 days.

Male Wistar rats were exposed to **SWCNT (CNI, HiPco)** by intratracheal instillation at 2.25 mg/rat (17.3 mg/kg) (Miyawaki et al.⁴⁶, 2008). Formation of foreign body granuloma in the lungs was observed. Severity of pathological findings was similar at 7 days and 90 days post-instillation (not recovered).

Pharyngeal aspiration

No information is available for principle SWCNTs, but there are seven studies for other SWCNTs as follows.

NIOSH (Shvedova et al.⁴⁷, 2005) demonstrated that pharyngeal aspiration of **SWCNT (CNI, HiPco)** elicited unusual pulmonary effects in female C57BL/6 mice that combined an acute inflammation with progressive fibrosis and granulomas. An early neutrophils accumulation followed by lymphocyte and macrophage influx, was accompanied by early elevation of proinflammatory cytokines followed by fibrogenic transforming growth factor. A rapid progressive fibrosis found in mice exhibited two distinct morphologies: 1) SWCNT-induced granulomas mainly associated with hypertrophied epithelial cells surrounding SWCNTs aggregates; and 2) diffuse interstitial fibrosis and alveolar wall thickening likely associated with dispersed SWCNTs.

Male and female Fischer 344 rats were exposed to **SWCNT (Helix Material Solution, CVD)** at 2 mg/kg by pharyngeal aspiration (Mangum et al.⁴⁸, 2006). Twenty-one days after aspiration, histopathological examination showed an indication of inflammation in the lung cells.

C57BL/6 mice were exposed to **SWCNT (CNI, HiPco)** at 10 and 40 µg/mouse by intrapharyngeal instillation (Li et al.⁴⁹ 2007). Aortic mtDNA damage was developed at 7, 28, and 60 days after exposure. A single intrapharyngeal instillation induced activation of HO-1 in the lung, aorta, and heart tissue in HO-1 reporter transgenic mice.

SWCNT (CNI, HiPco) was given by pharyngeal aspiration in C57BL/6 mice at a dose of 0 or 40 µg/mouse (Shvedova et al.⁵⁰ 2007; NIOSH). Inflammation, damage, and fibrosis were observed during 1 day to 3 months post-exposure. Followings are observed: rapid but transient inflammation and damage, rapid and persistent granulomas, and rapid and progressive fibrosis. Fibrosis is greater in vitamin E deficient mice and less in NADPH ox Kos

Pharyngeal aspiration of **SWCNT (CNI, HiPco)** in C57BL/6 mice at 0-20 µg/mouse caused inflammatory response, oxidative stress, collagen deposition, and fibrosis as well as mutations of *K-ras* gene locus in the lung of mice (Shvedova et al.⁵¹ 2008; NIOSH).

NIOSH (Mercer et al.⁵², 2008) investigated an effect of dispersion of **SWCNT (CNI, HiPco)**. Male C57BL/6 mice were exposed to SWCNT or dispersed SWCNT by pharyngeal aspiration at 10 µg/mouse. Lung sections and lavage cells demonstrated an early, transient neutrophilic and inflammatory phase that rapidly resolved and was similar to that observed with large agglomerates. No granulomatous lesions or epithelioid macrophages were detected. Dispersed SWCNT was rapidly incorporated into the alveolar interstitium and produced an increase in collagen deposition.

Dermal

Three acute skin irritation studies were conducted for **Nikkiso SWCNT** or **Super Growth SWCNT** according to OECD TG 404. No deaths or abnormal findings were observed at the maximum concentration that could be prepared (see 4.1.2 skin irritation for further detail).

Oral

An acute oral toxicity study was conducted based on OECD TG 423 (Matsumoto et al.⁵³, 2012). The maximum dose of 2000 mg/kg required by the guideline was impracticable because of very high specific volume of SWCNT. Three female Crl:CD(SD) rats were gavage dosed with **Nikkiso SWCNT** (suspended in 5% arabic-gum aqueous solution) at a total dose of 50 mg/kg bw (four equally divided doses at one-hour intervals). No deaths occurred, and no abnormalities were observed in the clinical

condition during the observation period in any animals. The LD₅₀ of Nikkiso SWCNT was considered to be greater than 50 mg/kg bw.

Two *in vivo* micronucleus studies are also available as follows.

Male Crlj:CD1(ICR) mice (6 animals/group) were gavaged with **Nikkiso SWCNT** (suspended in the water and was diluted with 0.3% CMC-Na solution) at 5, 10 or 20 mg/kg bw/day two times in the interval of 24 hours (OECD TG 474) (Naya et al, 2011). Male Crlj:CD1(ICR) mice (5 animals/group) were gavaged with **Super Growth SWCNT** (suspended in PBS with 1% Tween 80) at 60 or 200 mg/kg bw/day two times in the interval of 24 hours (NEDO [#NN-30] unpublished study). No death or indicative of abnormality is observed in both studies.

Studies in Humans

No information is available.

Summary

A single dose of SWCNT by intratracheal or pharyngeal aspiration caused inflammation of lung cells in rats or mice. An inhalation study (4days, 5h/day) also showed inflammation of lung cells in mice. The acute oral LD₅₀ of Nikkiso SWCNT was considered to be greater than 50 mg/kg bw in rats. No death was observed after two gavaged doses of 200 mg Super Growth SWCNT /kg bw/day in mice.

4.1.3 Irritation

Skin Irritation

Studies in Animals

Two skin irritation studies were conducted for **Nikkiso SWCNT** according to OECD TG 404. In one study, Nikkiso SWCNT solution (0.5g of 1 wt% in olive oil; maximum concentration that could be prepared) was applied three male Kbl:NZW rabbits of 17 weeks of age (Ema et al.⁵⁴, 2011; NEDO [#NN-40]). In the other study, Nikkiso SWCNT solution (0.5ml of 0.3 wt% in silicon oil) or powder of 0.02 g sopped with silicon oil was applied to each three male Kbl:JW rabbits of 10 weeks of age (NEDO [#NN-17]). No clinical signs or changes in body weight gain were observed in any groups treated with SWCNTs. No dermal responses, including erythema/eschar oedema, were found in rabbits.

Similarly, a skin irritation study was conducted for **Super Growth SWCNT** according to OECD TG 404. Super Growth SWCNT solution (0.5g of 1 wt% in olive oil; maximum concentration that could be prepared) was applied three male Kbl:NZW rabbits of 17 weeks of age (Ema et al.⁵⁴, 2011; NEDO [#NN-39]). No clinical signs or changes in body weight gain were observed in any groups treated with SWCNTs. No dermal responses, including erythema/eschar oedema, were found in rabbits.

Studies in Humans

No information is available.

Eye Irritation

Studies in Animals

Two eye irritation tests were done using Kbl:NZW rabbits for Nikkiso and Super Growth SWCNTs, following OECD TG 405 (Ema et al.⁵⁴, 2011; NEDO [#NN-35 and #NN-36]). Nikkiso SWCNTs solution

(0.1ml of 0.1 wt% in olive oil) or Super Growth SWCNTs solution (0.1ml of 0.5 wt% in olive oil) was dropped into the conjunctival sac of left eye of each three males. No clinical signs or changes in body weight gain were observed in any groups after the instillation of CNTs. Ocular responses, such as corneal opacity, conjunctival redness, abnormality of the iris, and chemosis, were not detected in rabbits at any observation period.

Studies in Humans

No information is available.

Respiratory Tract Irritation

Studies in Animals

Studies in Humans

No information is available.

Summary

Both Nikkiso and Super Growth SWCNTs were not irritating to the skin or eyes in rabbits.

4.1.4 Sensitisation

Studies in Animals

Skin

Two sensitization tests were executed using each 40 male Slc: Hartley guinea pigs (10 for the negative control, 10 for the positive control and 20 for material treated groups) for **Nikkiso and Super Growth SWCNTs**, following OECD TG 406 (Ema et al.⁵⁴, 2011; NEDO [#NN-43 and #NN-44]). No clinical signs or changes in body weight gain were observed in any group. No erythema or edema was observed after the challenge with Nikkiso and Super Growth SWCNTs. The skin sensitization experiments were properly performed, because positive dermal responses were observed in the area challenged with DNCB (positive control), but not in those areas challenged with acetone (negative control).

Respiratory Tract

No information is available.

Studies in Humans

No information is available.

Summary

Both Nikkiso and Super Growth SWCNTs were not sensitizing in guinea pigs.

4.1.5 Repeated Dose Toxicity

Studies in Animals

Inhalation

An inhalation test of **Super Growth SWCNT** was performed in Wistar rats for 4 weeks (6 hours/day, 5 days/week) at a level of 0.03 +/- 0.003 mg/m³ or 0.13 +/- 0.03 mg/m³ of the particle weight concentration in the exposure chamber (OECD TG 412) (Morimoto et al.⁵⁵, 2011). Inflammation and fibrotic response were examined at three days, one month or three months after the last exposure. Neither the low concentration exposure group nor the high concentration exposure group showed increase of the pulmonary wet weight, the infiltration of the inflammatory cell and increase of the HO-1 gene expression. An inhalation test for **Nikkiso SWCNT** has been conducting based on OECD TG 412 (NEDO [#NN-20] unpublished study). Wistar rats were exposed to Nikkiso SWCNT for 4 weeks (6 hours/day, 5 days/week) at a level of 0.40 +/- 0.11 mg/m³ of the particle weight concentration in the exposure chamber. Inflammation and fibrotic response were examined at three days, one month or three months after the last exposure. The study results are under evaluation.

Intratracheal

Super Growth SWCNT (suspended in PBS with 1% Tween 80) was administrated to male Crl:CD(SD) rats by intratracheal instillation for 5 times (once a week) at 0, 0.04 or 0.2 mg/kg (NEDO [#NN-08] unpublished study). BALF was examined at 1, 4, or 13 weeks after last instillation. Body weight and food consumption were not affected. Increases in white blood cells, eosinophils, proteins, LDH and IL-1 β were observed up to 13 weeks of the observation period at 0.04 and 0.2 mg/kg. At 0.2 mg/kg, increases in lung weight were observed up to 13 weeks. Histopathological examination revealed aggregation of macrophages at 0.04 and 0.2 mg/kg.

Pharyngeal aspiration

Repeated exposure to **SWCNT (CNI, HiPco)** was conducted at 20 μ g/mouse once every other week for 8 weeks by pharyngeal aspiration in ApoE^{-/-} transgenic mice (Li et al.⁴⁹, 2007) Although SWCNT exposure did not modify the lipid profiles of these mice, it resulted in accelerated plaque formation in ApoE^{-/-} mice fed an atherogenic diet. Plaque areas in the aortas, measured by the *en face* method, and in the brachiocephalic arteries, measured histopathologically, were significantly increased in the SWCNT-treated mice.

Dermal

No information available.

Oral

A repeated oral dose toxicity study was conducted based on OECD TG407. The maximum dose of 1000 mg/kg required by the guideline was impracticable because of very high specific volume of SWCNT (Matsumoto et al.³³, 2012). Male and Female Crl:CD rats (5 or 10 animals/sex/dose) were administered **Nikkiso SWCNT** (suspended in 5% guam acacia) by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). No treatment related changes of body weight, behavioral and blood biochemical parameters were observed. A few minor changes with statistical significance in white blood cells composition, organ weights and urine volume were detected, although no relevant pathological changes were observed. Based on the above

findings, the NOAEL of repeated oral dose toxicity of the SWCNT was considered to be 12.5 mg/kg bw/day (the highest dose tested) in rats.

Studies in Humans

No information is available.

Summary

Intratracheal instillation of Super Growth SWCNT (5 times, once/week) at 0.04 and 0.2 mg/kg showed indication of inflammation of the lung cells in rats. However, an OECD TG412 repeated inhalation toxicity study of Super Growth SWCNT for 4 weeks (6 hours/day, 5 days/week) showed no effects on the lung in rats. Results of a repeated inhalation toxicity study of Nikkiso SWCNT are under evaluation. Findings of this study will be useful to conclude repeated inhalation toxicity of SWCNT. A repeated oral dose toxicity study was conducted based on OECD TG407. Male and Female rats were administered Nikkiso CNT at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). Based on no toxicological effects, the NOAEL of repeated dose toxicity of the SWCNT was considered to be 12.5 mg/kg bw/day (the highest dose tested) in rats.

4.1.6 Mutagenicity

Studies in Animals

In vitro Studies

Bacterial mutation

A reverse gene mutation assay for **Nikkiso SWCNT** was performed using *S. typhimurium* TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA according to OECD TG471 at the concentration of 1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/plate (NEDO [#NN-22] unpublished study). Though no strains showed the growth inhibition without metabolic activation, cytotoxicity was observed for *S. typhimurium* TA100 and *E. coli* at 100 µg/plate, for *S. typhimurium* TA1535 and TA1537 at 50 and 100 µg/plate and for TA98 at 12.5 and 25µg /plate with metabolism activation. No mutation induction was observed with or without metabolic activation in all the tested concentration and in all the strain. It was judged that there was no increase of mutation frequency. Positive control showed expected levels of mutagenicity.

A reverse gene mutation assay for **Super Growth SWCNT** (suspended in 0.1% CMC-Na solution) was performed using *S. typhimurium* TA 97, TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA/pkM101 according to the Japanese Guideline (Chemical Substances Control Law of Japan) at the concentration of 12.5, 25, 50, 100, 200 and 500 µg/plate (Naya et al.⁵⁶, 2011; NEDO [#NN-01]). No mutation induction was observed with or without metabolic activation in all the tested concentration and in all the strain. Positive control showed expected levels of mutagenicity.

A reverse gene mutation assay for **SWCNT (CNI, HiPco)** was performed using *S. typhimurium* YG1024 and YG1029 without S9 mix at 0-240 µ g/plate (Kisin et al.⁵⁷, 2007). No increases in mutation frequencies in either YG1024 or YG1029 were found at all concentrations of SWCNT.

Chromosomal aberration

A chromosomal aberration test for **Nikkiso SWCNTs** (suspended in the water, sonicated and diluted with 0.3% CMC-Na solution) was conducted using cultured Chinese hamster lung (CHL/IU) cells according to

the OECD TG 473 (NEDO [#NN-26] unpublished study). The short term test (6 hours) with or without metabolic activation and the long term test (24 hours) with metabolic activation were performed at a concentration of 6.25, 12.5, 25 and 50 µg/plate. In either test condition, the expressions of structural chromosomal aberration and polyploidy were below 5%. The positive controls were effective for induction of chromosome aberrations.

A chromosomal aberration test for **Super Growth SWCNTs** (suspended in 0.1% CMC-Na solution) was conducted using cultured Chinese hamster lung (CHL/IU) cells according to the OECD TG 473 and Japanese Guideline (Chemical Substances Control Law of Japan) (Naya et al.⁵⁶, 2011; NEDO #NN-25). The short term test (6 hours) with or without metabolic activation and the long term test (24 hours) with metabolic activation were performed at a concentration of 300, 500 or 1000 µg/plate. In either test condition, the expressions of structural chromosomal aberration and polyploidy were below 5%. The positive controls were effective for induction of chromosome aberrations.

An in vitro mammalian cell micronucleus test for SWCNT (CNI, HiPco) was carried out with CHL (V79) cells at a concentration of 0, 12, 24, 48 or 96 µg/cm² for 24 h (Kisin et al.⁵⁷, 2007). No indications of chromosomal breakage and/or mitotic spindle damage were found.

Other data on in vitro chromosome aberration studies are summarized in Table 3.1.

Table 3.1 Summary of other in vitro chromosome aberration studies

Substance /Manufacture	Type of study	Cell	Concentration (exposure period)	Result	Reference
Non-functionalized SWCNT	Cytokinesis-block micronucleus (CBMN)assay	HDMEC (PromoCell)	25-150 µl/ml (68 h)	↑ Micronuclei at 25 and 50 µl/m. ↓ Proliferation potential (CBPI) of cells	Cveticanin et al. ⁵⁸ (2010)
Amide functionalized purified SWCNT	Cytokinesis-block micronucleus (CBMN)assay	HDMEC (PromoCell)	25-150 µl/ml (68 h)	↑ Micronuclei at 25-150 µl/m.	
SWCNT/Sigma-Aldrich	Cytokinesis-block micronucleus (CBMN)assay	Murine macrophage cell line RAW 264.7	0.01-100 µg /ml (24 h)	↑ Micronuclei at doses above 0.1 µg/ml	Migliore et al. ⁵⁹ (2010)
>50% SWCNT , ~40% other CNT/Sigma-Aldrich	Cytokinesis-block micronucleus (CBMN)assay	Human bronchial epithelial BEAS 2B cell	36-360 µg /ml (24, 48, or 72h)	Dose independent increases of micronucleated cells at three concentrations in the 48 h treatment. (The authors' conclusion is positive)	Lindberg et al. ⁶⁰ (2009)

DNA damage and/or repair

Genotoxicity of SWCNT (**EliCarb®**, **Tomas Swan**) was assessed in the FE1-Muta™Mouse lung epithelial cell line. SWCNT did not induce cell death within 24 hr at doses between 0 and 200 µg/ml or during long-term subculture exposure (576 hr) at 100 µg/ml. However, cell proliferation was slower with SWCNT exposure and a larger fraction of the cells were in the G1 phase. SWCNT did not increase the level of strand breaks, but significantly increased the level of FPG sensitive sites/oxidized purines (22 and 56%, respectively) determined by the comet assay. The mutant frequency in the *cII* gene was unaffected by 576 hr of exposure to 100 µg/ml SWCNT (Jacobsen et al.⁶¹, 2008).

An comet assay was conducted for SWCNT (CNI, HiPco) with CHL (V79) cells at concentration of 0, 24, 48 or 96 $\mu\text{g}/\text{cm}^2$ for 3 or 24 h (Kisin et al.⁵⁷, 2007). A 3-h SWCNT treatment led to DNA damage only at the highest SWCNT concentration. A 24-h treatment led to DNA damage in a concentration-dependent way. A 24-h exposure to 48 $\mu\text{g}/\text{cm}^2$ of SWCNT significantly increased the level of migrated DNA, tail length and olive tail moment by 2.25-, 1.76-, and 2.8-fold, respectively, while treatment with 96 $\mu\text{g}/\text{cm}^2$ SWCNT produced elevation in these parameters by 2.5-, 1.94-, and 3.4-fold, respectively. Other data on in vitro DNA damage and/or repair studies are summarized in Table 3.2.

Table 3.2 Summary of other in vitro DNA damage and/or repair studies

Substance /Manufacture	Type of study	Cell	Concentration (exposure period)	Result	Reference
SWCNT/NIST	Double strand breaks (DSB) assay	Normal human NM and malignant MM mesothelial cell	25 or 50 $\mu\text{g}/\text{ml}$ (24 h)	\uparrow H2AX phosphorylation	Pacurai et al ⁶² (2008)
	Comet assay	Normal human NM and malignant MM mesothelial cell	25 or 50 $\mu\text{g}/\text{ml}$ (24 h)	\uparrow DNA migration	
Non-functionalized SWCNT	Double strand breaks (DSB) assay	HDMEC (PromoCell)	0.5-30 $\mu\text{l}/\text{ml}$ (24 h)	\uparrow γ -H2AX foci	Cveticanin et al (2010)
Amide functionalized SWCNT/	Double strand breaks (DSB) assay	HDMEC (PromoCell)	0.5-30 $\mu\text{l}/\text{ml}$ (24 h)	\uparrow γ -H2AX foci	
SWCNT/COCO, Chinese Academy of Science	Comet assay	Primary mouse embryo fibroblast (BALB/c mouse)	5 or 10 $\mu\text{g}/\text{ml}$ (24 h)	\uparrow %Tail DNA \uparrow Tail length \uparrow Tail moment	Yang et al ⁶³ (2009)
SWCNT/Heji	Comet assay	Human leukocytes	1, 5 or 10 $\mu\text{g}/\text{ml}$ (6 h)	No effects	Zeni et al ⁶⁴ (2008)
>50% SWCNT , ~40% other CNT/Sigma-Aldrich	Comet assay	Human bronchial epithelial BEAS 2B cells	3.8-380 $\mu\text{g}/\text{ml}$ (24, 48 or 72 h)	\uparrow %Tail DNA at 3.8 $\mu\text{g}/\text{ml}$ and more	Lindberg et al (2009)

In vivo Studies

Chromosomal aberration

Nikkiso SWCNT, suspended in water and diluted with 0.3% CMC, was administrated twice by gavage in the interval of 24 hours to Crlj:CD1(ICR) mice of the age of 9 weeks (6 mice a group) at 5, 10 and 20 mg/kg/day (NEDO [#NN-30] unpublished study). In 24 hours, the bone-marrow cell was harvested from the femur and micronucleus assay was performed following OECD TG 474. No abnormality was seen in all the group of mice and no micronucleus formation was observed in each dose group of SWCNT.

Super Growth SWCNT, suspended in PBS with 1% Tween 80, was administrated twice by gavage in the interval of 24 hours to Crlj:CD1(ICR) mice of the age of 6 weeks (5 mice a group) at 60 or 200 mg/kg/day (Naya et al.⁵⁶, 2011; NEDO [#NN-29]). In 24 hours, the bone-marrow cell was harvested from the femur and micronucleus assay was performed following OECD TG 474. No abnormality was seen in all the group of mice and no micronucleus formation was observed in each dose group of SWCNT.

DNA damage and/or repair

Nikkiso SWCNT was dosed at 0.2 or 1.0 mg/kg once or 0.04 or 0.2 mg/kg for 5 times (once/week) by intratracheal administration in male Crl:CD (SD) rats (NEDO [#NN-33] unpublished study). A comet assay was conducted in lung tissue taken from 3 h (single dose and repeated dose) or 24 h (single dose) after administration. There were no effects on %tail DNA.

Instillation of **SWCNT (EliCarb® , Tomas Swan)** was conducted to ApoE^{-/-} mice at dose of 54 µg/mouse (Jacobsen et al.⁶⁵, 2009). A comet assay revealed significant increases in *Il-6*, *Mip-2* and *Mcp-1* mRNA in lung tissue at 3 h and 24 h following instillation of SWCNT.

Pharyngeal aspiration of **SWCNT (CNI, HiPco)** in C57BL/6 mice at 0-20 µg/mouse caused mutations of *K-ras* gene locus in the lung of mice (Shvedova et al.⁴¹, 2008; NIOSH).

C57BL/6 mice were exposed to **SWCNT (CNI, HiPco)** at 10 and 40 µg/mouse by intrapharyngeal instillation (Li et al.⁴⁹, 2007). Aortic mtDNA damage was developed at 7, 28, and 60 days after exposure.

The level of oxidative damage to DNA (8-oxodG) in the colon mucosa, liver, and lung of Fisher rats was assessed after gavage administration of **SWCNT (Thomas Swan)** at 0.064 or 0.64 mg/kg bw (Folkman et al.⁶⁶, 2009). SWCNT increased the levels of 8-oxodG in liver and lung.

Studies in Humans

No information is available.

Summary

Nikkiso and Super Growth SWCNTs did not induce gene mutation in bacterial *in vitro* tests (OECD TG 471 and Japanese guideline). Nikkiso and Super Growth SWCNTs did not induce chromosome aberrations in cultured Chinese hamster lung (CHL/IU) cells (OECD TG 473 and Japanese guideline). *In vivo* micronucleus assays (OECD TG 474) for Nikkiso and Super Growth SWCNTs showed negative results. Although a comet assay in lung tissues taken from rats given Nikkiso SWCNT intratracheally showed no effects on %tail DNA, many other studies (the comet assays, CBMN assays, DSB assays, *K-ras* mutation test, mtDNA assay and oxidatively damaged DNA assay) indicated possible DNA damage caused by SWCNTs.

4.1.7 Carcinogenicity

No information is available for carcinogenicity of SWCNT, but carcinogenic potential can be evaluated by an ongoing intratracheal instillation study with a two-year observation period (NEDO [#NN-14] unpublished study).

Summary

No information is available for carcinogenicity of SWCNT, but carcinogenic potential can be evaluated by an ongoing intratracheal instillation study with a two-year observation period (NEDO [#NN-14] unpublished study).

4.1.8 Toxicity for Reproduction

Studies in Animals

Pregnant CD-1 mice were intravenously injected with SWCNT, oxidized-SWCNT and ultra oxidized-SWCNT at 0 - 30 µg/animal on day 5.5 of gestation (Pietrojusti et al.⁶⁷, 2011). No deaths, clinically relevant disorders or evident behavioral changes were observed in all dams. Histological and immune-

histochemical analysis of maternal tissues revealed no toxicological effects on the liver, lung, kidney and spleen. A high percentage of early miscarriages or fetal malformations were observed in the oxidized SWCNT group, while lower percentages were observed in SWCNT group at 0.1 µg/animal and higher. All placentas from malformed fetuses appeared abnormal. The LOAEL of reproductive and developmental toxicity of SWCNTs was considered to be 0.1µg/animal.

Studies in Humans

No information is available.

Summary

Pregnant mice were intravenously injected with SWCNT, oxidized-SWCNT and ultra oxidized-SWCNT at 0 - 30 µg/animal on day 5.5 of gestation. In dams, no adverse effects were observed except in placenta. A high percentage of early miscarriages or fetal malformations was observed in the oxidized SWCNT group, while lower percentages were observed in SWCNT group at 0.1 µg/animal and higher. The LOAEL of reproductive and developmental toxicity of SWCNTs was considered to be 0.1µg/animal. Further study will be necessary to confirm reproductive and developmental toxicity of SWCNT.

4.1.9 Toxicity in vitro

In Germany, the neurotoxicity and gliotoxicity were studied by using several cell culture systems obtained from fetal or newborn rat brain for three types of SWCNTs (unpublished study, need further information).

*SWCNT (a.t.) :SWCNTs cleaned with acid in order to remove contaminations of metal-catalysts.

*fSWCNT (a.t.):SWCNTs cleaned with acid and functionalised with salmon sperm DNA.

*fSWCNT:SWCNTs functionalised with salmon sperm DNA but not acid treated .

Exposure of fSWCNT (a.t.) reduced the normal adhesion of OLN-93 cells. Exposure of SWCNT (a.t.) caused significant decrease of cell viability. fSWCNT (a.t.) caused significant increase of cytotoxicity in astrocytes and neurons. A decrease of metabolic activity in astrocytes was observed upon exposure of fSWCNT (a.t.), but not upon exposure of SWCNT. OLN-93 cells exhibited a strong reduction of cell proliferation upon exposure of fSWCNT (a.t.). Significant reduction of cell proliferation in astrocytes was found upon fSWCNTs (a.t.). Exposure to fSWCNTs (a.t.) significantly reduced the total cell numbers of astrocytes and OLN-93 cells. A reduction of the mitochondrial membrane potential was found upon exposure of fSWCNT (a.t.). Upon fSWCNT (a.t.) exposure, cells revealed a significant increase of intracellular calcium concentration. Stimulation of calcium by rIL-3 strengthened the effect. No increase of calcium concentration was found for fSWCNT of the identical exposure concentrations and for DNS alone.

Radomski et al.⁶⁸ (2005) demonstrated the ability of SWCNT (SES Research) to induce platelet aggregation and *in vivo* vascular thrombosis by using human platelet samples and carotid artery of Wistar-Kyoto rats.

Raja et al.⁶⁹ (2007) demonstrated the ability of SWCNT (CNI, HiPco) to alter aortic smooth muscle growth by using rat aortic smooth muscle cells.

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